# IDENTIFICATION OF DOMOIC ACID AS THE TOXIC AGENT RESPONSIBLE FOR THE P.E.I. CONTAMINATED MUSSEL INCIDENT

A summary of work conducted at the
Atlantic Research Laboratory of the
National Research Council, Halifax

13 December, 1987 to 11 January, 1988

Canada

ATLANTIC RESEARCH LABORATORY TECHNICAL REPORT 56
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# IDENTIFICATION OF DOMOIC ACID AS THE TOXIC AGENT RESPONSIBLE FOR THE P.E.I. CONTAMINATED MUSSEL INCIDENT: A SUMMARY OF WORK CONDUCTED AT THE ATLANTIC RESEARCH LABORATORY OF THE NATIONAL RESEARCH COUNCIL, HALIFAX, BETWEEN 13 DECEMBER, 1987, AND 11 JANUARY, 1988.

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## INTRODUCTION

Between November 11 and December 12, 1987, 156 cases of acute intoxication were linked to ingestion of cultured blue mussels (Mytilus edulis L.) that had been harvested from a localised area in eastern Prince Edward Island. Symptoms ranged from vomiting, abdominal cramps or diarrhea occurring within 24 hours of mussel consumption, to confusion, disorientation, loss of memory or other objective neurological signs within 48 hours. Of the 22 people hospitalised, 10 were admitted to intensive care units due to respiratory insufficiency, cardiovascular instability , or coma. Three elderly people died, and several others are still suffering long-term neurological effects. Details of the symptomology, which is very different from that typical of paralytic shellfish poisoning, have been described in:

T.M.Perl, L.Bedard, T.Kosatsky, J.Hockin and R.S.Remis, presented at the Epidemic Intelligence Service Conference, Center for Disease Control, Atlanta, Georgia, 19 April 1988.

Toxicity was detected in the cultured mussels by the Department of Fisheries and Oceans (DFO) using the standard methods of extraction/mouse bioassay prescribed for detection of paralytic shellfish poison (PSP) by the Association of Official Analytical Chemists:

"Official Methods of Analysis", Association of Official Analytical Chemists, Arlington, Virginia, (1984), 18.086 - 18.092.
See also: W.N.Adams and J.J.Miescier, J. Assoc. Official Analyt. Chemists 63, 1336 (1980).

Extracts of contaminated mussels injected intraperitoneally into laboratory mice produced an unusual scratching syndrome followed by death. These symptoms were again very different from those characteristic of PSP and all efforts to identify the toxic agent(s) responsible, through the first week of December, had failed.

On Friday, December 11th, it was agreed by Mr.Regis Bourque of the Department of Fisheries and Oceans (Chairman of the Mussel Toxin Working Group which had been organised to deal with the crisis) and Dr. R.A. Foxall, Director of the Atlantic Research Laboratory (ARL) of the National Research Council, that the concentration of expertise and specialised equipment for the isolation, identification and analysis of chemical compounds, located at ARL, would be dedicated to an intense effort to isolate and characterise the toxin(s). It was clear that success of this effort would depend upon bioassay-guided chemical analysis, requiring immediate access to mouse bioassay facilities. Accordingly DFO agreed to transfer an experienced operator of the mouse bioassay procedure (D.Richard), together with a mouse colony, from Black's Harbour, N.B., to ARL on December 12th.

No suitable laboratory-animal facility existed at ARL. However an intensive effort by the ARL Physical Plant Maintenance personnel transformed a laboratory into a space suitable for a laboratory-mouse colony.

This spirit of unstinted cooperation and camaraderie amongst all personnel involved from both ARL and DFO, including senior administrative staff, professional scientists, technical officers and maintenance and administrative support staff, was characteristic of this operation which succeeded in identifying the toxin within about 104 hours of round-the-clock effort. Indeed the considerable scientific success owed as much to this spirit, shown by all concerned who are named below, as to the scientific and technical skills employed.

The majority of the team was composed of ARL personnel, as reflected in the status of the present document as an ARL Technical Report. It is important, therefore, to acknowledge the essential contributions made by colleagues from other government departments. Essential to the progress of the project was the cooperation of the Department of Fisheries and Oceans, who provided the all-important mussel samples (L.Lea), expertise in mouse bioassay techniques (D.Richard), general knowledge concerning shellfish testing (M.Gilgan), acquisition of water and other marine samples (M.Bewers, P.Dickie, D.V.Subba Rao, F.Jodrey and P.Vass), and continuing discussions with senior scientists (R.Addison,

R.Bourque, S.MacPhee, J.Stewart and J.Worms). In addition the exchange of information with analytical chemists at the Department of Health and Welfare (H.Conacher, J.Lawrence, B.Lau,P.Scott) was of great value. Plankton-tow samples from the contaminated area were provided by Prof. L.Hanic, University of PEI.

This report is comprised largely of a summary of work completed at ARL over a period of approximately 4 weeks from 13 Dec., 1987 to 11 Jan., 1988. This summary was prepared for a joint meeting of the Mussel Toxin Working Group (comprising several government departments) held at Moncton, New Brunswick, on 12 Jan. 1988. As such it represents a snapshot of work accomplished at that time. The original objectives of the team at ARL were:

A to isolate and characterise the toxin(s) responsible;

B to develop procedures permitting reliable quantitative analyses;

C to study the origins of the toxin(s).

At the time that this summary was written Objective A had been essentially achieved, excellent progress towards Objective B had been made, and a start had been made for Objective C. Since that time considerable further progress has been made but, since work is still continuing at the time at which this report was prepared for issue as an ARL Technical Report (July 1988), these new results will be reported separately.

Towards the end of the period of time covered in the present report, it became apparent that a considerable body of knowledge was available concerning the pharmacology of toxins related to domoic acid, the compound eventually identified as the responsible toxin. Since no relevant expertise was available at ARL, it was necessary to seek help elsewhere and we were fortunate to acquire the services of Dr.Thomas White of Dalhousie University as an NRC consultant. His commissioned report is included as an Appendix; this was important at the time since it confirmed that the known symptomology was consistent with the identification of domoic acid as the toxic agent.

The division of the investigation into categories A, B and C is convenient for purposes of discussion, but is otherwise somewhat artificial and is not adhered to rigorously in what follows.

MEMBERS OF THE TEAM WORKING ON THE ARL CONTAMINATED MUSSEL PROJECT, 13 DECEMBER 1987 TO 11 JANUARY 1988.

NATIONAL RESEARCH COUNCIL.

Halifax.

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ARL Technical Officers and Temporary Workers: C.A.Craft, E.W.Dyer, D.J.Embree, M.G.Flack, C.Gillis, M.Greenwell, W.R.Hardstaff, P.LeBlanc, N.I.Lewis, G.K.McCully, M.McInerney-Northcott, D.O'Neil, P.F.Seto and D.Tappen.

ARL Maintenance and Administrative Support Staff: K.Gray, W.Gray, B.Garside, H.O.Henderson, K.MacLeod, D.Robson, G.Saab, M.Schofield, C.Vaughan, H.Watts and B.Willis.

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Division of Chemistry: J.W.McLaren.

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Dept. of Biology: L.Hanic.

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Preparation of this report was coordinated by R.K.Boyd.

July 6, 1988.

## A. ISOLATION AND IDENTIFICATION OF TOXIN(S).

1. Several different batches of contaminated mussels, plus one "control" batch of non-toxic mussels, were examined in parallel. This was an essential feature of the project.

2. It was established (J.McLaren, Chemistry Division, NRC, Ottawa) that the heavy metal content of both toxic and control mussels was entirely normal, and that no significant levels of the common man-made xenobiotics (pesticides, PCBs, PAHs, etc.) were present (ARL, GC/MS method).

3. Initial efforts were directed to organic extractables. Although a false trail, HPLC "fingerprints" of these organic extracts did show striking differences in the pigment patterns between toxic and non-toxic mussels. In future work these differences may prove useful in identification of seston material which may have acted as vector for the toxin, or even its ultimate source.

4. Strict control was established over the doses used in the mouse bio-assay, to ensure that each dose could be related to a known weight of original mussel tissue (referred to below as the "g.equiv. of tissue injected"). This was essential for tracking the toxicity throughout the fractionation, and for estimation of toxin recovery and enrichment factors.

5. It was established that the toxin(s) was (were) water-soluble. Negligible toxicity (mouse bio-assay) was found in organic layers in partitioning experiments.

6. Since the nature of the toxin(s) was completely unknown at this point, as was the stability towards strong acid or base, it was decided to use a mild extraction procedure (aqueous methanol at room temperature) rather than more severe procedures (e.g. boiling HCl, as used in standard methods for paralytic shellfish poisons). This particular aqueous methanol method later turned out to be non-quantitative (but see #B 3 below), but was ideal for the initial qualitative identification step because less interfering material was co-extracted.

7. Organic/aqueous partitioning removed large amounts of material (e.g. carotenoids, lipids, etc.) into the organic layer, leaving the toxicity in the aqueous layer (see #A 4 above). This clean-up procedure helped in the initial stages of the investigation.

8. Chromatography on an XAD open column (aqueous mobile phase) removed almost all of the less-polar lipid material remaining in the room-temperature aqueous methanol extract. The toxicity was concentrated in the first two fractions collected. Subsequent organic washes of the column recovered no additional toxicity.

9. A crude dose/response curve, relating time-of-death (TOD) to weight-equivalent of mussel tissue extracted to provide the bioassay test injection, was obtained early on (Figure 1). This indicated a very narrow dynamic range (factor of only about 8 in time of death (TOD)) for the mouse bio-assay of the principal toxin(s). This finding implied that strict control of dose level (see #A 4 above), within the range of 1.2 to 2.5 g equiv. of original mussel tissue, was essential for the following reasons:

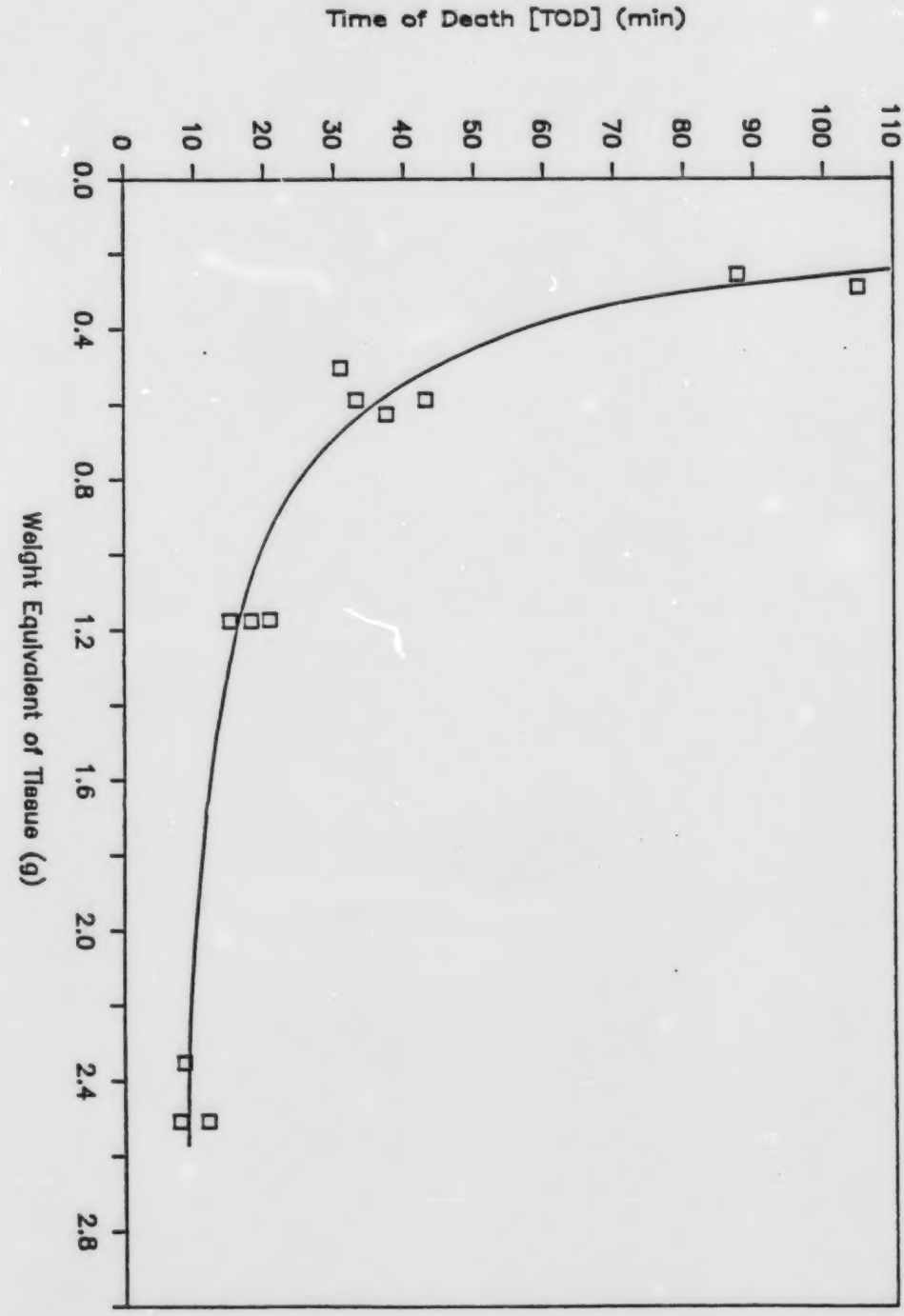
(a) since there may be more than one toxin, so that the separation procedure would result in distributing the toxicity amongst several fractions, some or all of the toxicity could thus become undetectable if too low a weight equivalent of tissue were used;

(b) a similar false-negative result could arise even if only one toxin were present, if the separation procedures tried were such as to distribute the toxin over several fractions;

(c) too high a value of weight equivalent of tissue dose, e.g. via too high a concentration factor applied to any fractionated material, would result in false-positive results due to toxic reaction to minor amounts of substances which are non-toxic at their naturally occurring levels --- indeed this was the source of the problem (see #A 3 above)

Figure 1. Crude dose-response curve relating time-of-death to weight-equivalent of mussel tissue (wet weight) used to provide the extract injected for the bioassay.

FIGURE 1


Dose Response for Mouse Bioassay
Time of Death [TOD] (min)
0
10
20
30
40
50
60
70
80
90
100
110
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
Weight Equivalent of Tissue (g)

in early erroneous results, which had indicated that the toxin was organic soluble.

10. It was important to establish more than one chromatographic procedure, for both fingerprinting and fraction isolation. By thus employing two such techniques with very different separatory characteristics, it was possible to perform meaningful crosschecks on conclusions drawn from isolation of toxic fractions. The technique first successfully applied in the toxic mussel investigation at ARL was high-voltage paper electrophoresis (HVPE), which has separatory characteristics very different from those of reversed phase HPLC (see #A 11 below), for example.

HVPE has the advantage that it separates the extract into acidic, neutral and basic components. By cutting the chromatogram into sections, followed by extraction of the separate paper sections with distilled water, it was determined that all of the toxicity moved towards the anode at pH 6.5; this indicated that the toxin(s) carried an excess of acidic groups (e.g. excess of carboxyl over amino or imino functional groups). Toxicity was entirely absent from the corresponding chromatograms for control mussels. By repeating this experiment, but cutting more narrow sections, it was determined that the toxic activity migrated on the trailing edge of glutamic acid. The broad band, corresponding to glutamate, stained red with ninhydrin and partially masked any trailing band(s). Later, after fractions isolated by preparative HPLC (see #A 12 below)

were further analysed by HVPE, it was possible to establish that the toxicity was associated with a yellow band (ninhydrin reaction) no longer obscured by the red band due to glutamic acid (removed by the HPLC procedure). Finally preparative HVPE on the raw aqueous methanol extract, rather than on an XAD fraction, showed that no detectable toxicity occurred other than in the band corresponding to the toxic band described above. Unfortunately the HVPE electrophoretograms do not lend themselves to reproduction by photocopying, and thus are not illustrated here.

11. Fingerprinting of appropriate XAD fractions (#A 8 above) was also achieved using reversed-phase HPLC with diode-array detection (DAD), which provides capability for complete UV spectra for each eluting component. The reasons for choosing reversed phase HPLC were based upon the known polarity and water solubility of the toxin(s); the detailed method that proved successful was originally selected on the premise that the toxin was a hydrophilic peptide. Complete experimental details are given in ARL Technical Report #55, ("Determination of domoic acid in shellfish tissue by HPLC"). A particular cluster of chromatographic peaks (one major, several minor) was present in the HPLC/DAD trace (Figure 2, shown for absorption at 210 nm) of all toxic (mouse bio-assay) fractions but not in those from control samples. The UV spectrum (Figure 3) of the major peak

Figure 2. Comparison of HPLC traces for corresponding fractions of extracts of toxic and control mussels. Chromatograms for absorption at 210 nm were computer-reconstructed from complete UV spectral data stored on disk.

FIGURE 2

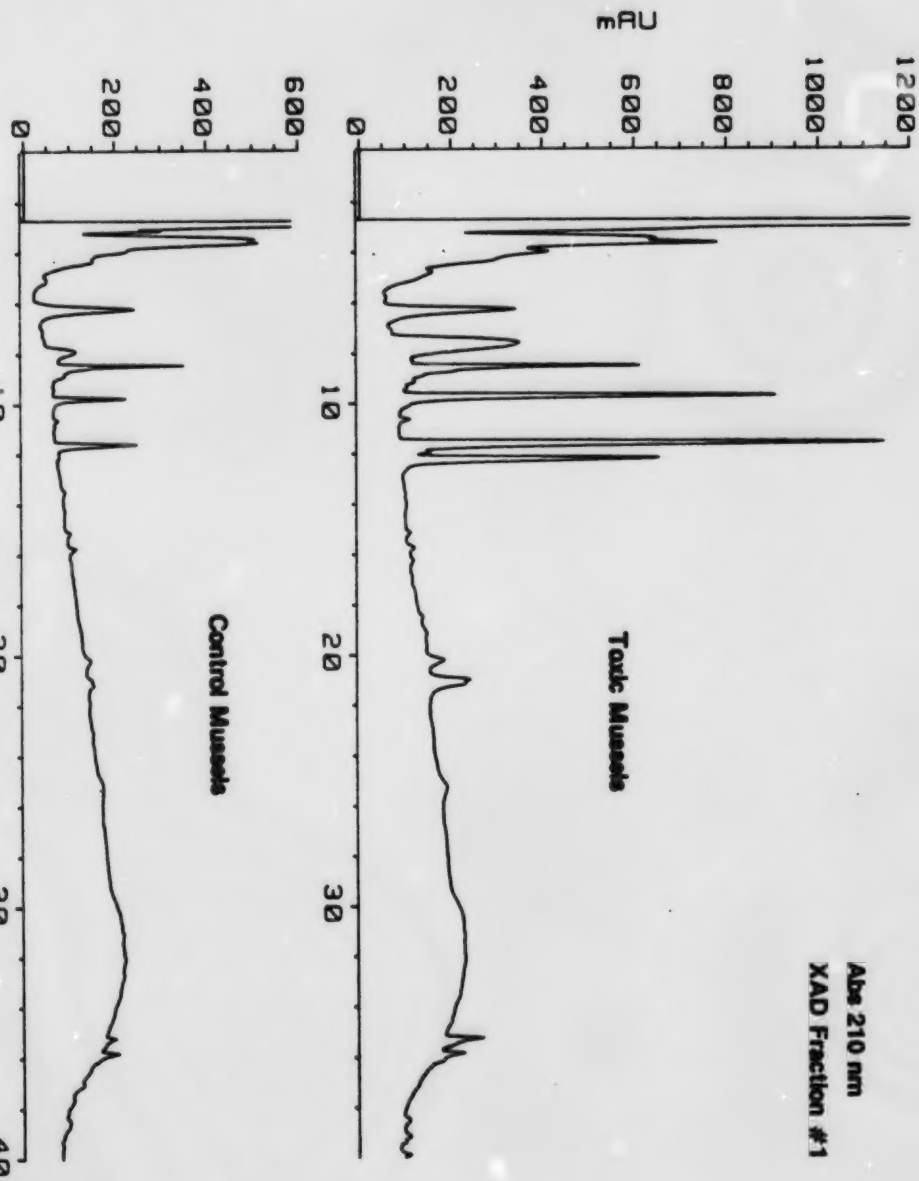

Abs 210 nm
XAD Fraction #1
Toxic Mussels
Control Mussels
mAU
Time (min.)
0
200
400
600
800
1000
1200
10
20
30
40

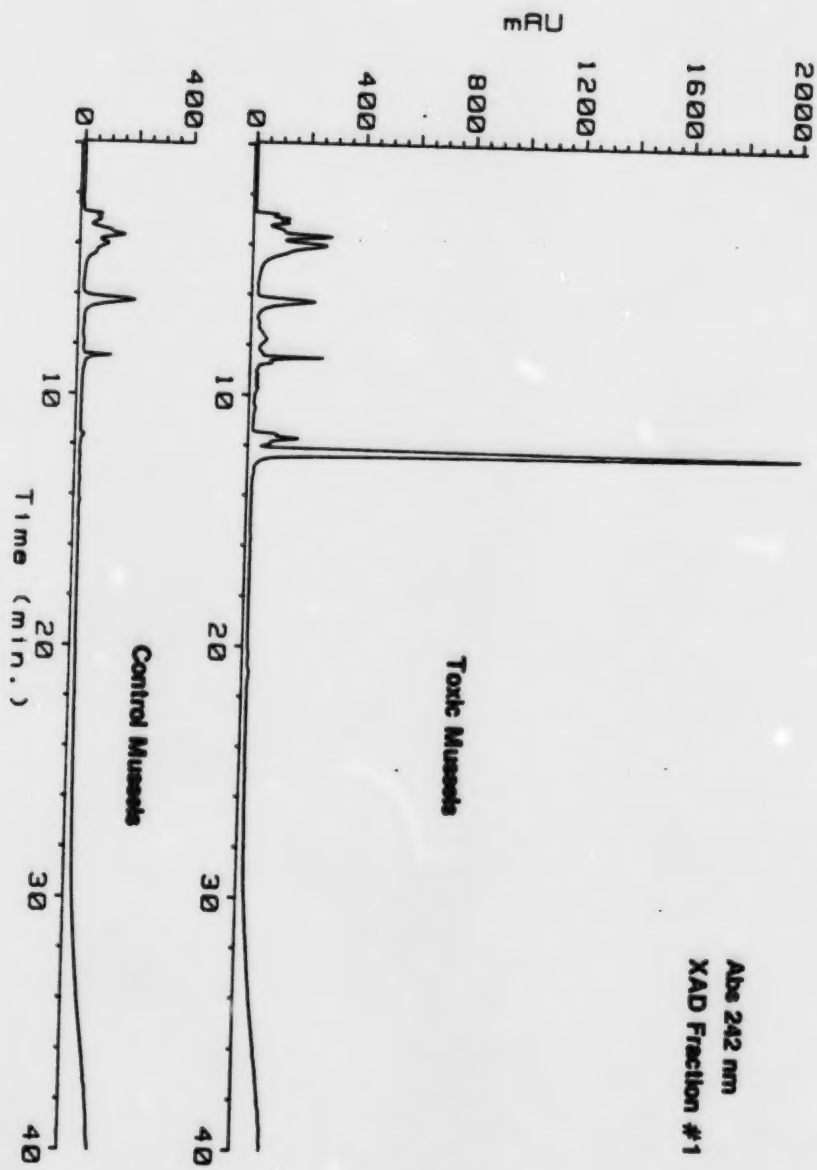
Figure 3. Absorption spectrum for compound eluting at about 12.5 min. (Figure 2) for toxic, but not for control, mussel extracts.

FIGURE 3

mAU
0
40
80
120
160
200
220
240
260
280
300
Wavelength (nm)
$\lambda_{max}$ - 242 nm
Log $\epsilon_{max}$ - 4.42

Figure 4. Comparison of HPLC traces for corresponding fractions of extracts of toxic and control mussels. Chromatograms for absorption at 242 nm were computer-reconstructed from the sama data-set as was used to produce Figure 2.

FIGURE 4


mAU
2000
1600
1200
800
400
0
400
0
10
20
30
40
Time (min.)
Control Mussels
Toxic Mussels
Abs 242 nm
XAD Fraction #1

eluting at 12.5 min. (Figure 2) showed a broad absorption band peaking at 242 nm. When the chromatogram for absorption at 242 nm was computer-reconstructed from the DAD data (stored on disk), the difference between control and toxic mussels was greatly accentuated (Figure 4).

12. It was now possible to carry out a preparative HPLC procedure on the raw aqueous methanol extract (no XAD column), isolating successive fractions of the eluent for mouse bio-assay and spectroscopic characterisation. It was determined that all of the toxicity in this room-temperature aqueous methanol extract, to within limits imposed by the limits of precision for the mouse bioassay, was associated with the cluster of HPLC peaks mentioned in #A 11 above. This result was also consistent with that drawn from the corresponding HVPE experiment (#A 10 above).

13. Crosschecks between the two chromatographic techniques were now possible. For example the toxic band from the HVPE analysis (#A 10 above) was redissolved and subjected to the HPLC procedure. The retention time and UV spectrum indeed matched those originally found in the HPLC analysis (#A 11 above), though the resolution of the HVPE separation was such that the toxic fraction was contaminated with other compounds (the HPLC readily separated the toxin from these, especially glutamic acid). The converse experiment was also performed, with similarly satisfying results; since the HPLC fraction was free of other components, it could be established that the toxin did indeed run close to the

glutamic acid band in the HVPE analysis, and yielded a yellow colour on reaction with ninhydrin (see #A 10 above). This last observation suggests the presence of an imino group in the toxin molecule. Since the physical bases of the two chromatographic techniques are so different (electrophoretic mobilities vs. partition coefficients between aqueous and hydrophobic phases), the possibility of a misleading conclusion regarding the origin of the toxicity seemed remote.

The overall separation scheme used at ARL is summarised in Figure 5.

14. Preliminary characterisation of this major toxic fraction was now possible.

(a) The UV spectrum (from HPLC/DAD, #A 11 above) showed a broad absorption band peaking at 242 nm (Figure 3), suggesting extended conjugation but not of the conventional aromatic type.

(b) A strong cation-exchange column (Dowex 50, $H^+$ form, sulphonated polystyrene) indicated that the toxin(s) behaved as a cation at low pH and probably contained an amino or imino group.

(c) Positive-ion fast atom bombardment (FAB) mass spectra (Figure 6) of toxic extracts consistently showed a peak at m/z 312, absent from spectra of control samples. The

Figure 5. Summary of separation scheme used at ARL for isolation of mussel toxin. Open and full circles denote fractions which were found to be non-toxic and toxic, respectively. Half-filled circles symbolise fractions carrying marginal toxicity, defined in terms of a g.equiv. of wet tissue injected in the bioassay (see text).

FIGURE 5

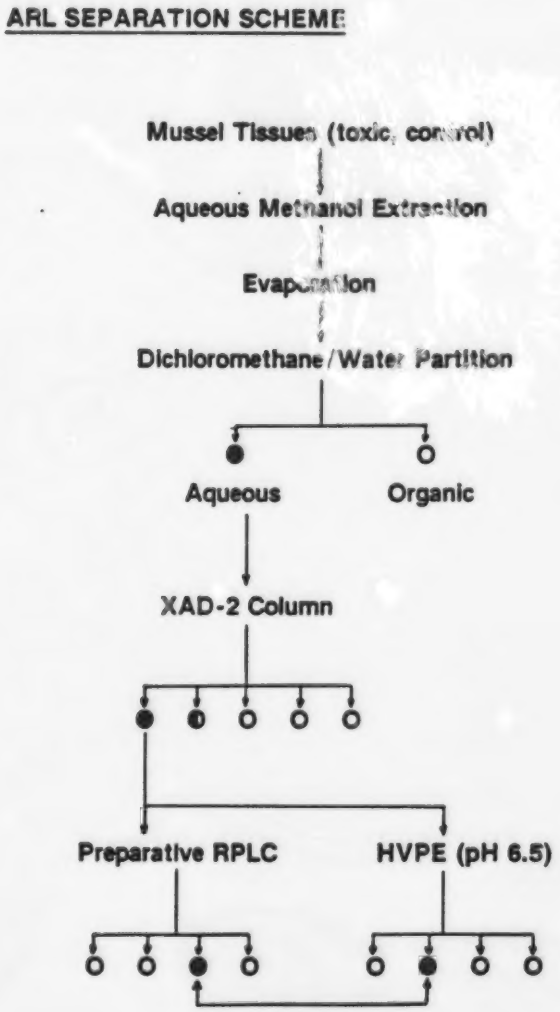
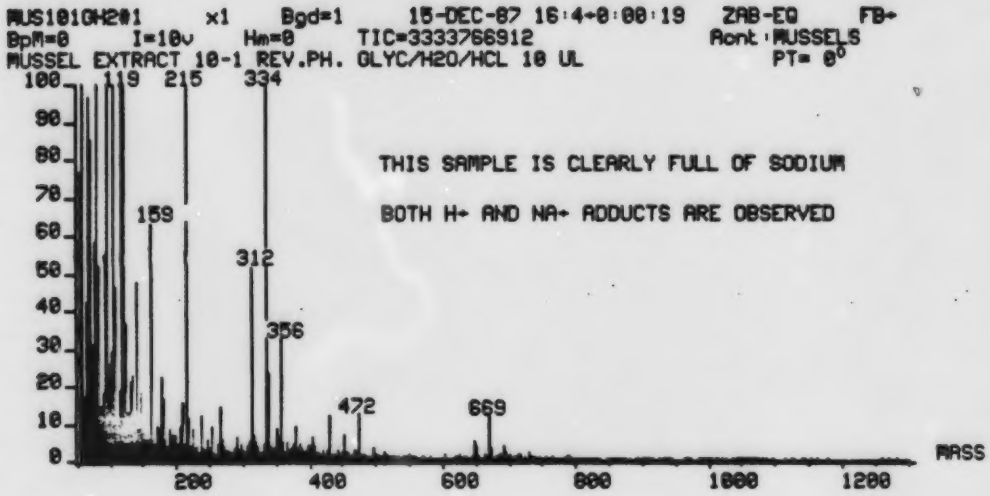

ARL SEPARATION SCHEME
Mussel Tissues (toxic, control)
Aqueous Methanol Extraction
Evaporation
Dichloromethane/Water Partition
Aqueous
Organic
XAD-2 Column
Preparative RPLC
HVPE (pH 6.5)

Figure 6. Positive-ion fast-atom-bombardment (FAB) mass spectra of a toxic mussel extract at different stages of purification. Matrix was a glycerol/water/HCl mixture.

FIGURE 6


MUS101GH2#1 x1 Bgd=1 15-DEC-87 16:4-0:00:19 ZAB-EQ FB-
BpM=0 I=10v Hm=0 TIC=3333766912 Acnt MUSSELS
MUSSEL EXTRACT 10-1 REV.PH. GLYC/H2O/HCL 10 UL PT= 0°
119
215
334
159
312
356
472
669
THIS SAMPLE IS CLEARLY FULL OF SODIUM
BOTH H+ AND NA+ ADDUCTS ARE OBSERVED
MASS

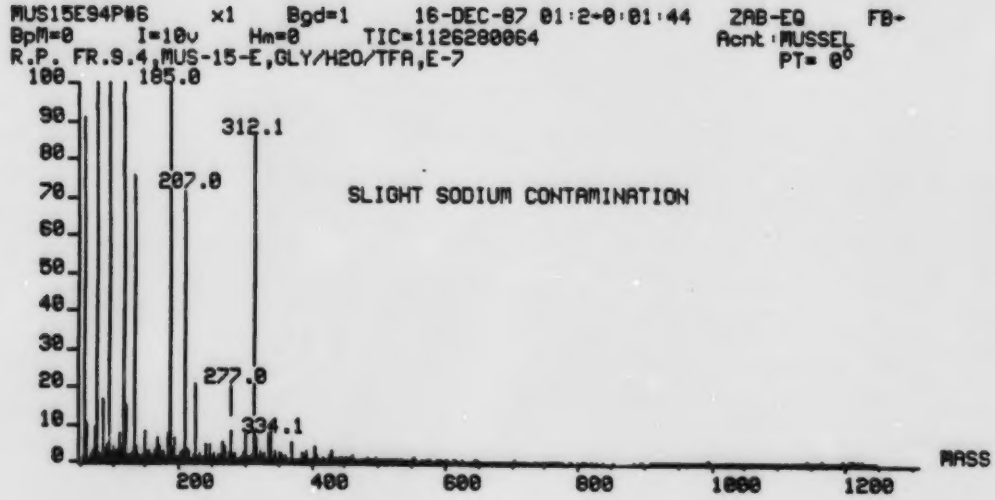
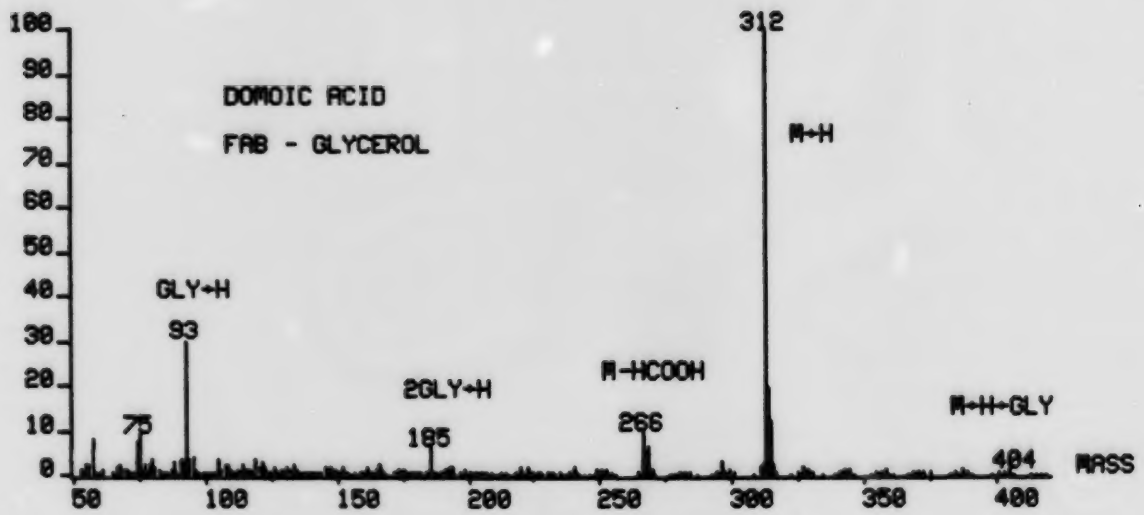

MUS15E94P#6 x1 Bgd=1 16-DEC-87 01:2-0:01:44 ZAB-EQ FB-
BpM=0 I=10v Hm=0 TIC=1126280064 Acnt MUSSEL
R.P. FR.9.4,MUS-15-E,GLY/H2O/TFA,E-7 PT= 0°
185.0
312.1
207.0
277.0
334.1
SLIGHT SODIUM CONTAMINATION
MASS

Figure 7. Positive-ion FAB mass spectrum of a highly purified sample of mussel toxin.

FIGURE 7


DOMOIC ACID
FAB - GLYCEROL
312
M+H
GLY+H
93
2GLY+H
185
M-HCOOH
266
M+H+GLY
404
MASS
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400

relative importance of this peak increased as the purity and toxicity of the fractions increased (Figure 7), and negative-ion FAB spectra confirmed that a molecular species of molecular weight 311 was present.

(d) Both NMR and FT-IR preliminary spectra yielded strong hints about chemical functional groups. For example, an olefinic proton signal observed in the NMR spectrum was consistent with an IR band characteristic of a trans -CH=CH- double bond. Similar evidence for at least two distinguishable carboxyl groups, and also for an imino functionality, was also obtained.

15. Further characteristics of this toxic HPLC fraction from the aqueous methanol extract were determined as follows:

(a) Conventional IEX-based amino-acid analysis, with and without acid hydrolysis, showed that the major toxin is not composed of the common amino acids, but confirmed the presence of a constituent which eluted between methionine and isoleucine (Figure 8), and which yielded a yellow ninhydrin derivative.

(b) The compound was stable to digestion by papain. This observation did not necessarily rule out the possibility that the toxin was a peptide.

(c) Ultrafiltration resulted in the toxicity being retained by a 500 Da filter but passed through a 1000 Da ultrafiltration membrane. The tentative assignment of a molecular weight in the range 500-1000 Da turned out to be misleading, but this procedure is useful from a preparative

point of view as it can be used to remove proteins and salts.

(d) The toxicity was stable towards hydrolysis by dilute strong acid or base, including the conditions (boiling dilute HCl) used for extraction of paralytic shellfish poisons (PSP). However later information (see #B 3 below) indicated that the situation was not as simple as at first believed.

(e) Accurate mass measurement (312.146 $\pm$ 0.003, peak matching method) of the ions of m/z 312 from the toxic fraction was sufficiently precise, when combined with information on functional groups from the tandem mass spectrometry experiments (see below), to tie down unambiguously the composition of the protonated molecule $(M+H)^+$ to be $C_{15}H_{22}NO_6$. The only other even-electron ion consistent with this information is $C_{12}H_{26}O_6NS$. In principle the isotopic intensity distribution should clearly distinguish between these possibilities (100:17.4:2.7 and 100:14.1:6.6, for the 312, 313 and 314 peaks of the two possibilities). However, while the intensity of the peak at m/z 313 was observed to fall in the range 16 - 19% of that of the peak at m/z 312 (thus favouring the the $C_{15}H_{22}NO_6$ formula), the intensity of the peak at m/z 314 was highly variable (in the range 4 - 25%), for even highly purified samples. Subsequent work has shown that this phenomenon

Figure 8. Response obtained for a standard mixture of amino acids plus purified mussel toxin (domoic acid, DA) using an IEX-based amino acid analyser.

FIGURE 8

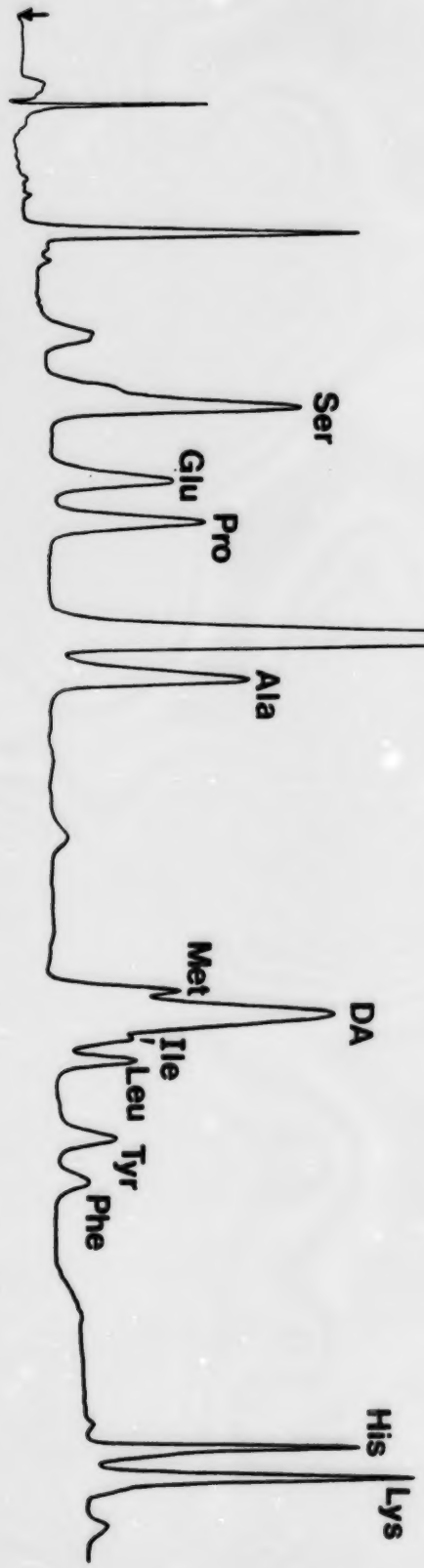
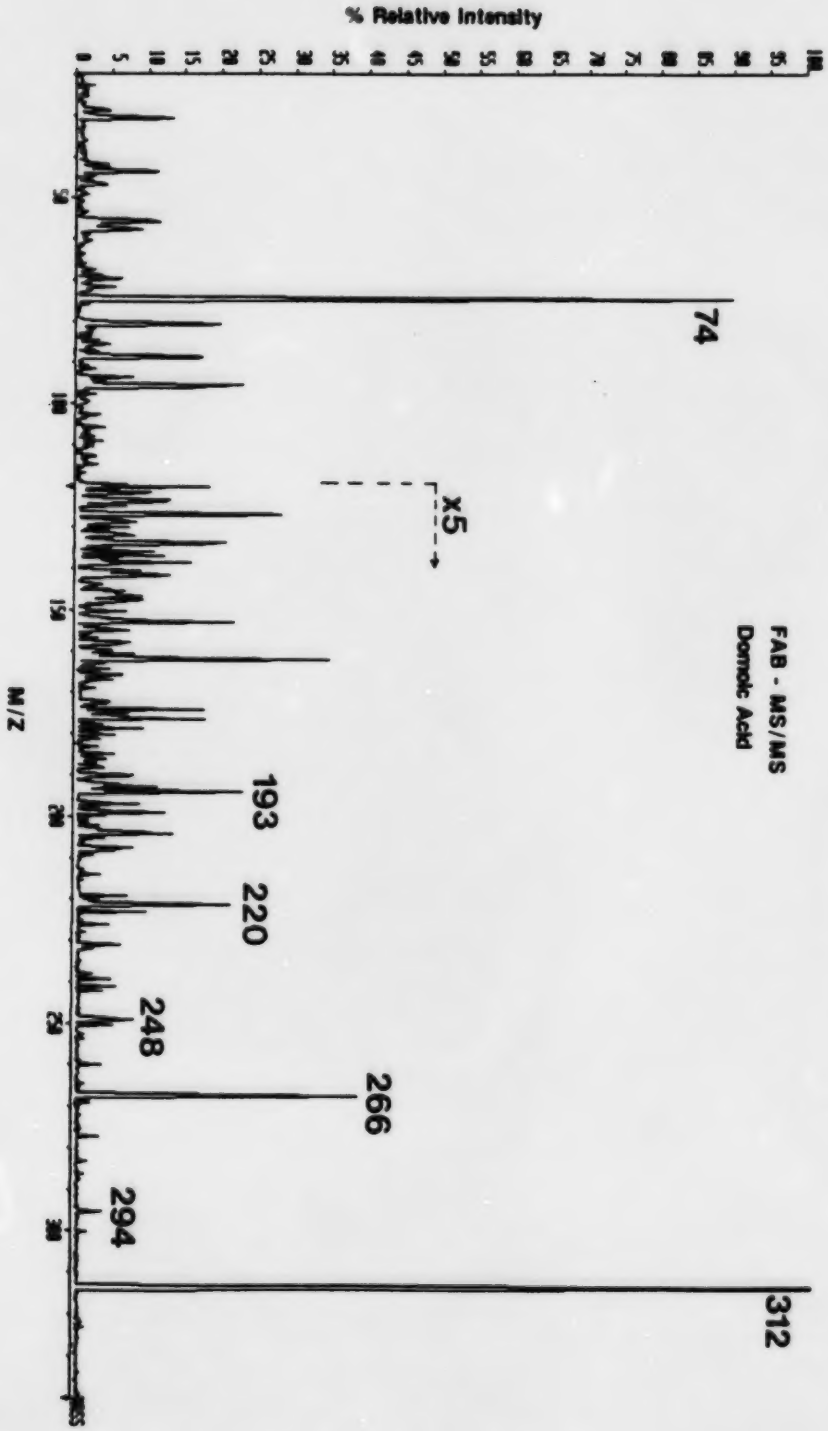

IEX Amino Acid Analysis
(Ninhydrin yellow)
Time (min)
Ser
Glu
Pro
Gly
Ala
Met
DA
Ile
Leu
Tyr
Phe
His
Lys

Figure 9. Fragment-ion spectrum of the ions at m/z 312, formed by FAB ionisation of a non-purified extract of toxic mussels (Figure 6). Laboratory collision energy was 30 eV, argon collision gas.

FIGURE 9


% Relative Intensity
FAB - MS/MS
Domoic Acid
M/Z
74
x5
193
220
248
266
294
312

corresponds to reduction of the analyte by the matrix under irradiation by the fast atom beam, rather than to variable proportions of a dihydro impurity which was not separable from the toxin.

The fragment ion spectrum of the ions of m/z 312, from an unpurified toxic fraction, is shown in Figure 9; the successive losses of 46 da (assigned as HCOOH) indicate the presence of at least two carboxyl groups and thus at least four oxygen atoms; the intense fragment ion at m/z 74 is known to be characteristic of a protonated amino acid (assigned as the ion $COOH-CH=NH_2^+$). No fragmentations characteristic of sulphur-containing functionalities were observed. The composition of the unprotonated toxin molecule was thus shown to be $C_{15}H_{21}NO_6$.

(f) Prolonged hydrolysis by more concentrated strong acid, however, destroyed the toxicity of this fraction. Positive ion FAB mass spectra of this hydrolysate now showed several peaks, of which easily the most intense was still at m/z 312; accurate mass measurement of this peak gave the same result as that for the unhydrolysed toxin (#Δ 15(e) above). However the fragment-ion spectrum of this ion (determined by tandem mass spectrometry) was significantly different from that of the isobaric ions formed by FAB ionisation of the toxic fraction, though the qualitative indications of two carboxyl groups were still present. This observation suggested that the toxin was in part converted by the severe hydrolysis to non-toxic isomers of the toxin. This

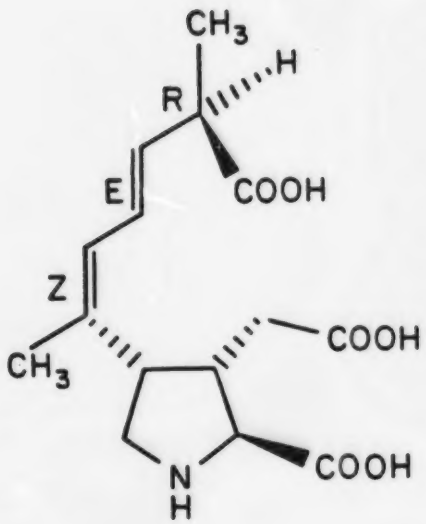
conclusion was supported by HPLC analysis of the hydrolysate; the original toxin peak (#A 11 above) was no longer present, but was replaced by several peaks eluting on either side of the toxin. Several of these presumed isomer peaks also showed the characteristic UV absorption at 242 nm.

16. As these data were obtained concerning the chemical nature of the toxin, they were used to search the chemical (Chemical Abstracts) and toxicological databases (D. Dewar, CISTI, Ottawa). The first such search was based upon only the information summarised in #A 14 above (viz. UV spectrum, nominal-mass molecular weight, and indications of functional groups from NMR and FT-IR spectroscopies) together with the symptomology of the toxin. As a result domoic acid (see structure, Scheme 1) was proposed as the most likely candidate amongst known compounds (Ref.11, Appendix 1), although several other candidate compounds could not be excluded at this stage. However, as the information became more detailed (#A 15 and 17) these other candidate compounds were progressively eliminated. These database searches undoubtedly speeded up the successful identification of the toxin by as much as two days or so.

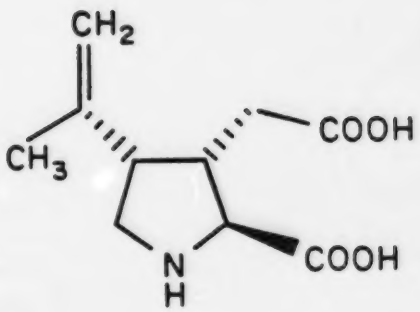
17. Simultaneously a major effort at ARL produced enough of the material contained in the HPLC peak associated with the

Scheme 1. Structures of the related marine neurotoxins domoic acid and kainic acid.

SCHEME 1




DOMOIC ACID




KAINIC ACID

Figure 10. Proton magnetic resonance spectrum (300 MHz) of a highly purified sample of mussel toxin. The assignments shown are identical with those published previously (Ref.11, Appendix 1) for domoic acid produced by an absolute synthesis.

FIGURE 10

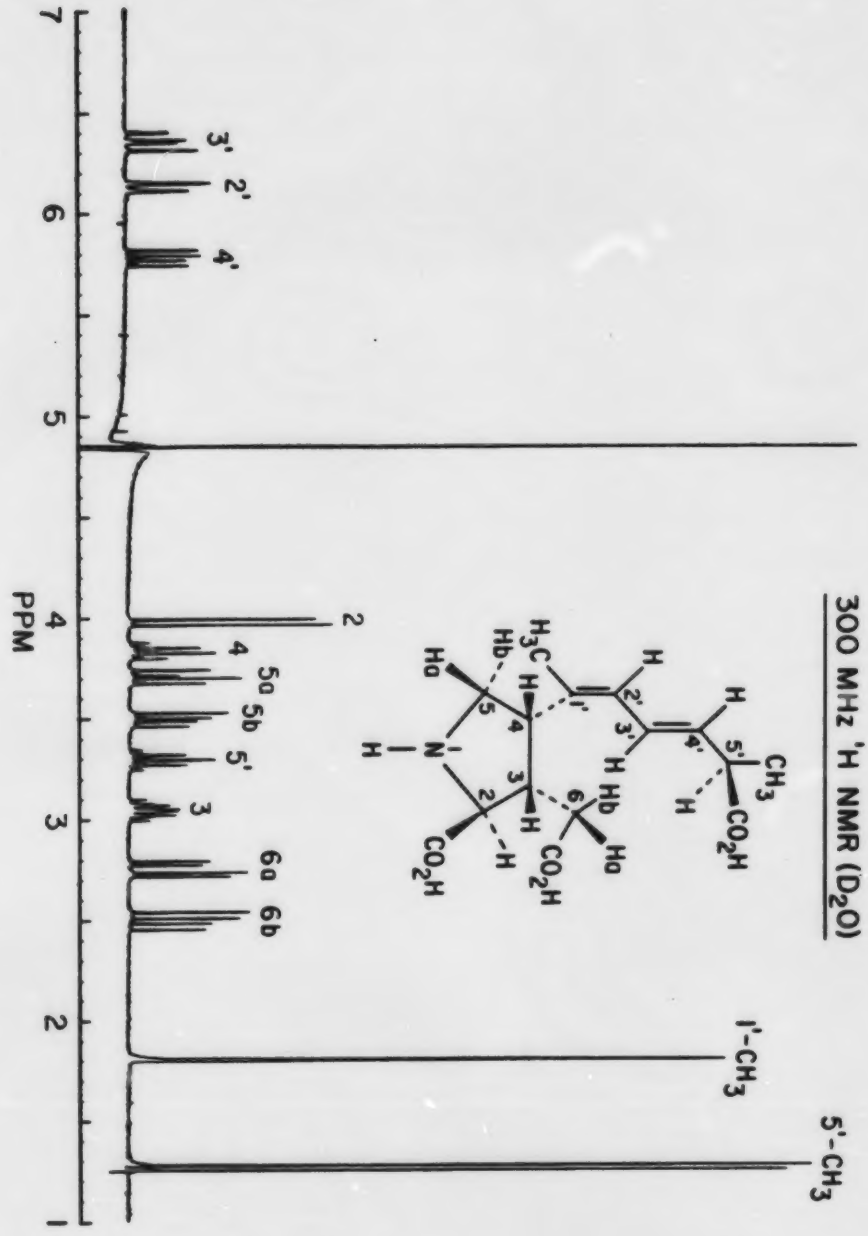


Figure 11. Comparison of Fourier transform infrared spectra of mussel toxin and of a commercial sample of domoic acid extracted from Chondria armata.

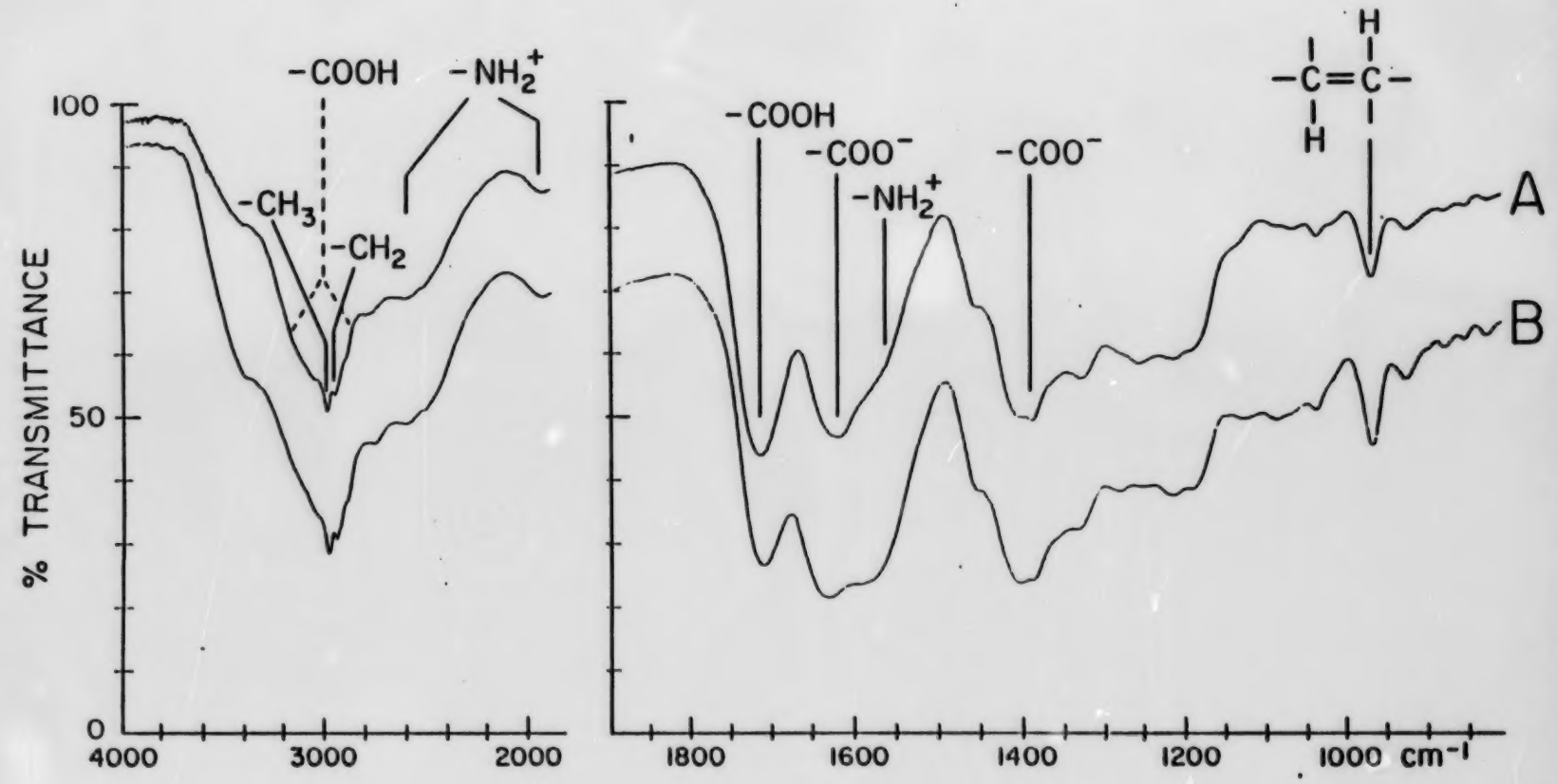



FIGURE 11

FT-IR SPECTRA OF SOLID FILMS ON $BaF_2$

A: FROM P.E.I. MUSSELS

B: AUTHENTIC DOMOIC ACID

toxicity that a good quality proton NMR spectrum (Figure 10) could be obtained. Both chemical shifts and scalar coupling constants were determined, and found to be indistinguishable from those published previously (Ref.11, Appendix 1) for synthetic domoic acid. Moreover, the nature of the NMR information was such that it was difficult to imagine any other chemical structure giving the observed spectral characteristics. Carbon-13 NMR spectra of the mussel toxin and of commercial domoic acid (obtained later) were closely comparable, although the quality of the spectra was limited by the small sample sizes available at this time. Small variations in chemical shift and linewidth of some peaks were entirely attributable to differences in pH, which was not adjusted at this time as there was a reluctance to contaminate either sample.

18. The major toxin in the aqueous methanol extract had now (between 3 and 4 p.m., Thursday 17th Dec.,1987) been identified as domoic acid. As a result it was possible to rationalise the other information (#A 14 and #A 15 above) on this basis. In particular details of the FT/IR spectrum (Figure 11) were successfully accounted for. It was now relatively straightforward to show that domoic acid was also the major toxin extracted using the PSP procedure (boiling dilute HCl).

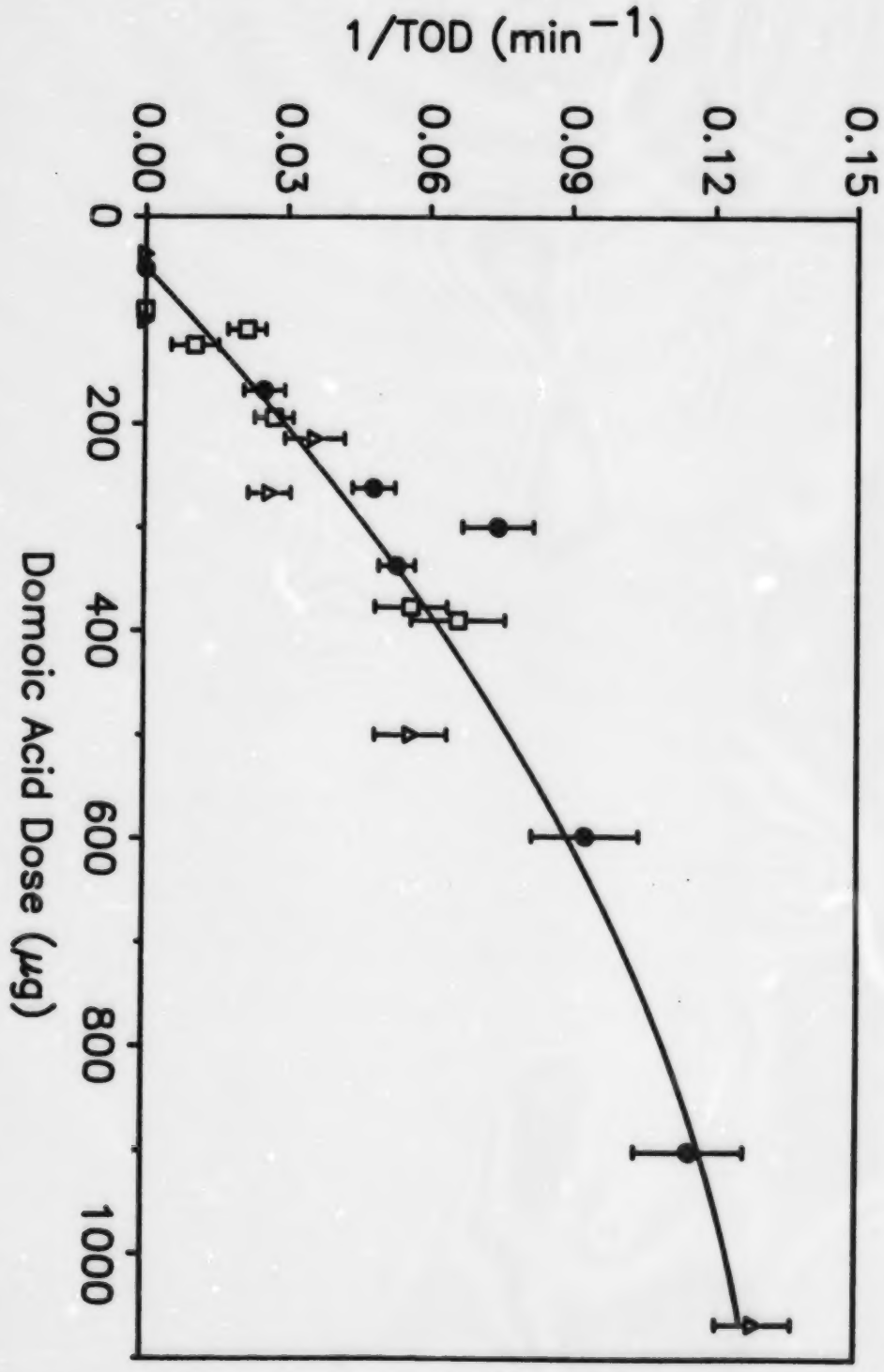
19. It was important now to investigate whether any toxins other than domoic acid were present at significant levels.

Two general approaches to this question were adopted---one based on quantitative and the other on qualitative analyses.

The quantitative approach is described in category B below, and is discussed here only with respect to the results which were important in the context of excluding other toxins. The general idea was to determine whether there exists any level of toxicity (mouse bio-assay) in the whole mussel extract which cannot be accounted for by the measured levels of domoic acid in the extract. The latter could now be accurately assayed by HPLC methods (see #B 1 and 2 below). Definite conclusions from this approach were limited by the intrinsically limited reproducibility of the mouse bio-assay and by effects of co-extracted material. (As an example of the latter effect, some raw aqueous methanol extracts were less toxic per unit of domoic acid than the domoic acid fractions isolated from them! Similar "protective" effects are well known in PSP bio-assays.) Nonetheless there was no evidence from these quantitative studies (Figure 12) that any significant toxicity other than that due to domoic acid occurs in the aqueous methanol extracts; this conclusion was valid to within the reproducibility of the mouse bio-assay. The dose-response curve for the PSP-type extracts (Figure 12) was also the same, within the experimental reproducibility, as that for purified domoic acid and for the aqueous methanol extracts.

Figure 12 Toxicity, expressed as reciprocal of time of death (TOD) of test mouse, as a function of the amount of domoic acid (as determined by HPLC) injected into the peritoneum. The toxicities of mussel extracts, both room temperature aqueous methanol (open triangles) and boiling 0.1 N HCl (PSP extraction method, open squares), are similar to that of aqueous solutions of purified domoic acid (full circles). Each point is the mean of between 2 and 4 replicate measurements of TOD for each solution; the error limits represent estimates of the standard deviations. The curve is a result of a least squares regression fit of all mean values to a second order polynomial.

FIGURE 12


1/TOD (min$^{-1}$)
0.00
0.03
0.06
0.09
0.12
0.15
0
200
400
600
800
1000
Domoic Acid Dose (µg)

Therefore any additional toxin could only have an effect whose magnitude is limited to values within the scatter of the bioassay data.

20. The search for other toxin(s) by qualitative analysis followed two somewhat complementary routes.

The first involved fractionation of raw mussel extracts by HPLC (and also by HVPE where possible) with toxicity testing of each fraction collected. As described above, the only toxic fraction thus found in the room-temperature aqueous methanol extracts was that associated with domoic acid (both HPLC and HVPE fractionation). Analogous screening of the dilute HCl raw extracts (standard methodology for PSP tests) was possible for HPLC fractionation only, since the amount of extraneous material thus extracted was found to overload the HVPE paper. The HPLC eluent of each of the PSP extracts (one toxic sample, one control) was collected as eight fractions, of which one (fraction 6) corresponded to the "cluster" associated with domoic acid (#A 11 above -- of course this cluster was missing from the control sample). Some additional toxicity was observed in fraction 4, which contained the bulk of the total extracted material, but the corresponding fraction from the control sample displayed similar potency and symptoms. In particular, when each fraction was diluted so that the domoic acid fraction caused death of the test mice in 15-20 min. (lower limit of the dynamic range, Figure 1), the toxicity of the correspondingly diluted fraction 4 was marginal, i.e. not

all mice died and the time of death for those that did was very long and irreproducibly so, with no significant difference between toxic and control samples. It was therefore concluded that the toxicity of fraction 4 was a non-specific effect, associated simply with the large quantity of extracted material in this fraction.

The second qualitative strategy in the search for additional toxins was to strip out the domoic acid from raw mussel extracts and then assess the toxicity of the residue. The stripping was done using a weak anion exchange column, a method with the obvious disadvantage that any additional toxin possessing characteristics similar to those of domoic acid would also be stripped out by this column. (The related (but much less potent) toxin kainic acid (Scheme 1) was shown to elute well before domoic acid under the HPLC conditions used, and to not be present in the toxic extracts). These stripping experiments were conducted for HCl extracts from both toxic and control mussels, by two independent operators using slightly different resins (competing brands), i.e. four different stripped extracts were subsequently tested for toxicity. The four were indistinguishable from one another in that all were equally toxic to mice, with identical symptoms which were very different from those characteristic of domoic acid. (Note that it is quite possible, unless considerable precautions are taken, to have some breakthrough of domoic acid from these columns). A blank experiment in which an appropriate solution of NaCl in 0.1 M HCl had its pH adjusted and was

then run through the anion exchange column, in exactly the same manner as the mussel extracts, produced death of test mice with the same symptoms.

These results clearly required further investigation. Accordingly the domoic-acid-free eluents from the columns (one from toxic mussels, one control mussels), both of which killed mice as described above, were subjected to ultrafiltration using a 500 da filter. The four resulting fractions were then tested for toxicity by the same strict quantitative regimen (#A 4) as was used throughout. Neither residue (not passing through the filter) was toxic to mice. The ultrafiltrates (which must have contained all salts, buffers, etc), however, were both toxic, with equal potencies for toxic and control mussels. Recall (#A 14(d)) that identical ultrafiltration of the raw mussel extracts showed that all the toxicity is retained by a 500 da filter.

These combined results do not conclusively prove that no other toxin is present, but probably come as close to this desirable result as is realistically possible.

21. Further characterisation of the "cluster" of peaks associated with domoic acid in the HPLC trace (#A 11 above) was necessary, to identify the minor components. It was possible to achieve a fairly clean HPLC cut of the minor peaks on either side of the main (domoic acid) peak. The toxicity of these satellite peaks was immeasurably low, when expressed in terms of toxicity (time-of-death) per gram equiv. of mussel tissue extracted for the injection; while

these additional constituents therefore cannot account for the vast majority of the toxicity of the mussels, they may possess intrinsic toxicity (i.e. per unit mass of pure compound) comparable to that of domoic acid itself. Work on this aspect is continuing. With respect to chemical characterisation of these satellite HPLC peaks, the best experiment conducted thus far (Jan.1988) involved collection of sub-fractions across this cluster, with off-line mass spectrometry of each sub-fraction. While some suggestive results were obtained (peaks at m/z 312 in the positive-ion FAB mass spectra) this procedure is not satisfactory, and arrangements have been made with other laboratories to analyse samples by suitable techniques of on-line HPLC/mass spectrometry. No results are available at this time.

## B. QUANTITATIVE ASPECTS OF EXTRACTION AND ANALYSIS OF DOMOIC ACID.

Chronologically the development of a reliable quantitative assay for domoic acid preceded the investigations of extraction efficiency from mussel tissue, although in the earliest stages these two aspects were not separable from one another.

1. A high-speed HPLC/DAD method for confirmation and quantitation of domoic acid has been developed. Clean-up of the raw tissue extract is performed using $C_{18}$ solid-phase extraction cartridges. After suitable elution and dilution, the sample is injected directly into the HPLC. The ARL work used a Hewlett Packard Model 1090 HPLC equipped with a diode array detector (DAD). A Vydac 201TP column of 2 mm i.d. is used, with isocratic elution by 0.5 ml/min of water with 10% acetonitrile plus 0.1 % trifluoroacetic acid. These conditions provide a 5-minute analysis with domoic acid eluting at about 3.2 min (Figure 13). The DAD is set to acquire at 242 and 210 nm simultaneously, and to acquire complete UV spectra either in the peak-triggered mode for routine screening or in the continuous mode for very important samples. Early versions of this fast extraction/cleanup procedure gave a recovery of 80% or so, but recent improvements have increased this to essentially 100%. The HPLC conditions developed at ARL have been adapted by the group at the Department of Health and Welfare to be more compatible with procedures used for PSP analysis; this

compatibility was achieved at the expense of a longer analysis time, but this seems likely to be an invaluable contribution. Details of the ARL methodology for analysis of shellfish for domoic acid are given in ARL Technical Report 55 (NRCC No. 29015).

2. The Beer-Lambert Law response curve for UV (242 nm) detection of domoic acid (Figure 14) shows excellent linearity with an intercept which is zero to within experimental uncertainty; the latter observation indicates negligible on-column losses at trace levels. Detection limits for a 5 microlitre injection volume lie in the range 0.3-1.0 ng, depending on the detector used (Figure 15). Details are included in the ARL Technical Report 55 (see #B 1 above). Confirmation of compound identity and quantitation is currently (Jan. 1988) performed using the IEX-based Amino Acid Analyser (Beckman Instruments Model 119CL, see Figure 8), which however has a detection limit for domoic acid of 200 ng (100 microlitre injection volume). A more sensitive confirmatory technique is thus required, and appropriate on-line HPLC/mass spectrometry may provide this.

3. A systematic study of extraction efficiencies for domoic acid from mussel tissue has been undertaken. Although not complete by mid-January, some interesting trends were

Figure 13. Example of the HPLC chromatogram (absorption at 242 nm) obtained as the final step of the fast analytical procedure, for domoic acid in shellfish, developed at ARL.

FIGURE 13

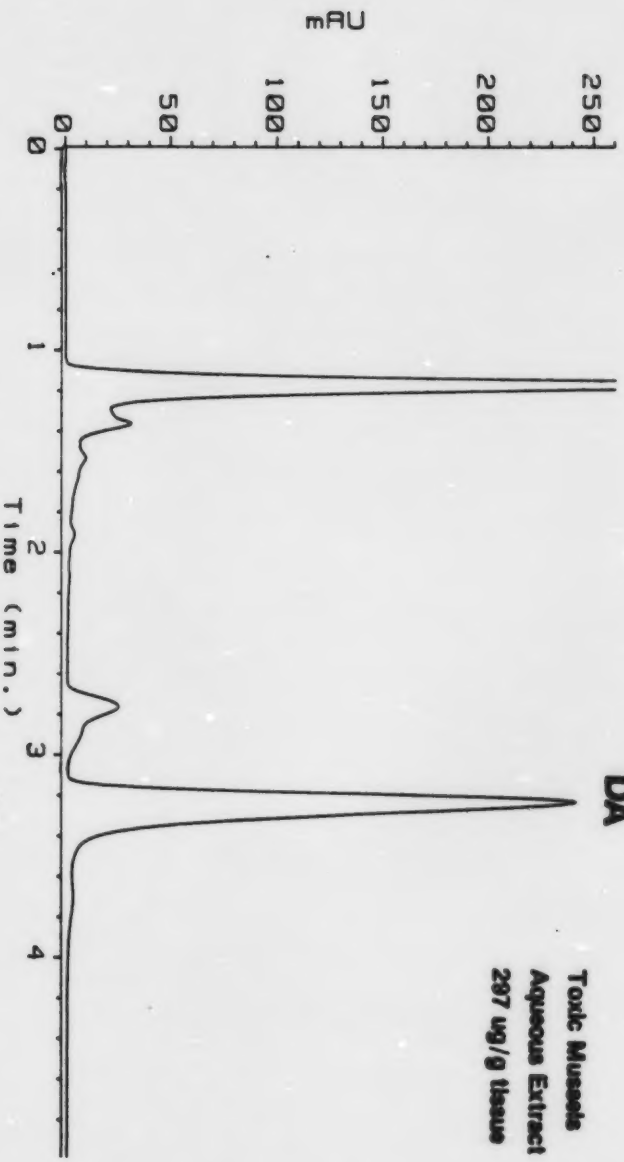

mAU
250
200
150
100
50
0
0
1
2
3
4
Time (min.)
DA
Toxic Mussels
Aqueous Extract
297 ug/g tissue

Figure 14. Calibration curve (Beer-Lambert Law) for domoic acid analysis by HPLC monitored by absorption at 242 nm.

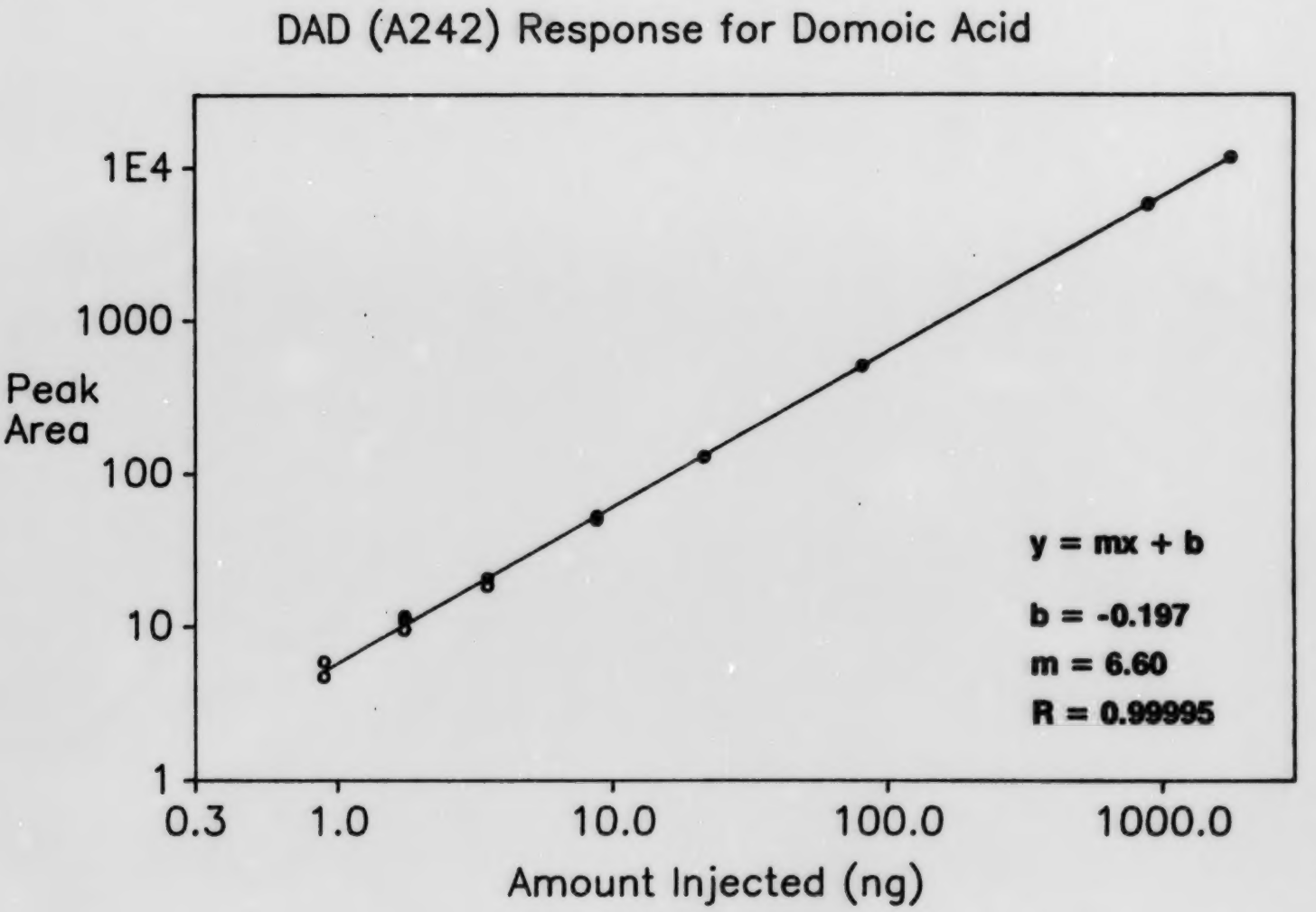
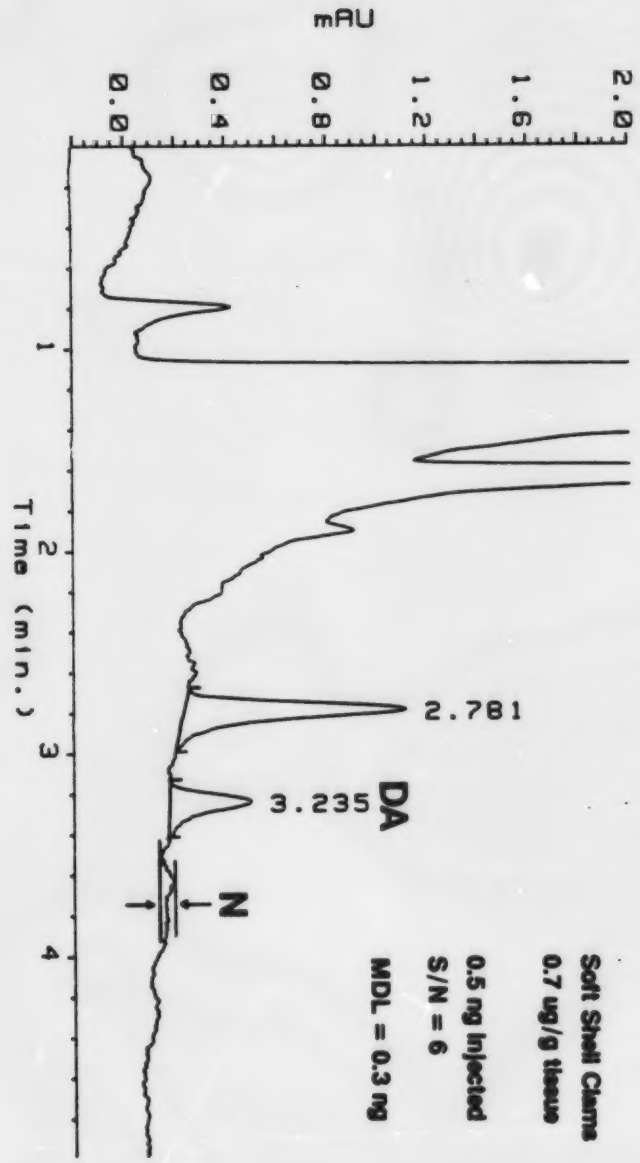

DAD (A242) Response for Domoic Acid
1E4
1000
100
10
1
Peak Area
y = mx + b
b = -0.197
m = 6.60
R = 0.99995
0.3
1.0
10.0
100.0
1000.0
Amount Injected (ng)

FIGURE 14

Figure 15. Example of domoic acid analysis by the fast ARL method, near the detection limit.

FIGURE 15


mAU
0.0
0.4
0.8
1.2
1.6
2.0
1
2
3
4
Time (min.)
2.781
3.235
DA
N
Soft Shell Clams
0.7 ug/g tissue
0.5 ng Injected
S/N = 6
MDL = 0.3 ng

already evident from this study. The extraction procedures which have been compared thus far (Jan.1988) are:
(i) PSP extraction (boiling 0.1 M HCl, 6 min.);
(ii) neutral aqueous extraction, room temperature;
(iii) aqueous methanol extraction, room temperature;
(iv) boiling aqueous methanol extraction, 5-10 min.
Some combinations of the foregoing have also been investigated. All results quoted below were obtained for aliquots of the same homogenised mussel tissue, and figures are in micrograms domoic acid per gram of wet homogenized tissue: (ii) (17 hours), 245; (iii) (24 hours) 240 and 243 in two different runs, with about 95% of the total successfully extracted after 4 hours; (iv) (10 min.), 240. The reproducibility within this set of four experiments is excellent.

Separate experiments showed that almost all the domoic acid is in the digestive gland, with almost none in the meat itself.

The PSP-type extraction (i) gave some unexpected results; the domoic acid content of this extract was only 70-75% of that obtained using methods (ii)-(iv), but subsequent extraction of the tissue residue using (iii) yielded only an additional 5% or so. The missing 20-25% of domoic acid could not be extracted or detected subsequently. A possible clue to understanding this discrepancy was obtained by monitoring extracts, obtained by method (i), for domoic acid content as a function of time of storage at room temperature after extraction. After 10 hours the domoic acid content (UV

assay) of the PSP-type extract had decreased by some 15%; a further 25% was lost after 7 days. This trend was not affected by neutralisation of the extract to pH 7 before cold storage. Note that standard solutions of purified domoic acid are stable for weeks under identical storage conditions, and that extracts obtained using methods (ii)-(iv) are certainly not unstable to any comparable degree. Recall however that the toxicity of PSP-type extracts is the same as that of aqueous methanol extracts when expressed in terms of domoic acid content of the extracts (Figure 12). Confirmation of these results, and further work on these phenomena, are in progress.

4. The solubility of domoic acid in aqueous and other media will clearly be highly pH-dependent. As a first step in the investigation of this and other phenomena which are likely to control bio-availability, extraction efficiencies, etc., literature values (Ref 1, Appendix 1) of the $K_a$ values for domoic acid have been used to calculate the distribution of the various ionic species as a function of pH. For example, the proportion of the zwitterionic form is thus calculated to be at a maximum at pH 2.9.

## C. ORIGINS OF DOMOIC ACID IN PEI.

Some preliminary and thus far (11 Jan. 1988) uninterpreted information has been obtained on this question. The main achievement has been to acquire water and plankton-tow samples from pertinent areas before winter freeze-up. Credit for most of these collections must go to L.Hanic (University of PEI) and to the Department of Fisheries and Oceans (coordinated by M.Bewers), though ARL personnel were involved in the first major collection of a water sample from a suspect area. A summary of observations made thus far at ARL is presented below.

1. Prior to the recent events on the east coast of Canada, the only documented sources of domoic acid were the original discovery (Ref.1, Appendix 1) in Chondria armata in Japan, and a later identification (Ref.6, Appendix 1) in another (Mediterranean) species of the family Rhodomelaceae, Alsidium corallinum. It was clearly necessary to inquire whether any related species were likely to be found in or near the affected areas.

By the time (mid-December, 1987) the identification of domoic acid had been made, it was too late to search for such algae in the waters around PEI. However, the ARL herbarium contains specimens of Rhodomelaceae including Chondria baileyana, which had been collected from PEI locations over a period of 25 years. Several such species, plus one non-Rhodomelacean red alga that occupies the same

ecological niche as C. baileyana, were analysed for domoic acid using both the ARL procedure (#B 1 above) and the amino acid analyser.

In the herbarium specimens, domoic acid was found only in Chondria baileyana, at levels of about 0.2% expressed in terms of dried weight of the residue after extraction. Of course this evidence is of questionable value in the context of the present problem, particularly in view of the surprisingly large quantities of domoic acid which must have been present. (From production records, about 63,000 kg of harvested mussels were affected. These contained domoic acid at levels as high as 900 micrograms per gram of wet tissue. Thus extremely high quantities of domoic acid must have been produced in the rather small affected area over a period of a few weeks).

The genus Chondria is rare in the southern Gulf of St. Lawrence where it is confined to estuaries and lagoons. Never abundant in even these habitats, it is unlikely to have occurred in sufficient quantity to toxify so many mussels. This is confirmed for 1987 by inspection of mussels collected in several PEI locations, including one of the affected areas, throughout the summer and fall of 1987; these collections were the work of A.L.Mallet and C.E.A.Carver of the Bedford Institute of Oceanography (DFO). No evidence was found of Chondria growing on these mussel shells or associated culture netting, either as basal discs of the past summer's plants or as young stages of next summer's growth.

2. A large sample (approx. 10,000 litres) of water was collected using a portable pump in a small boat from one of the affected areas, and transported to ARL. This was maintained at 0-5 deg.C (but not frozen), and continuous centrifugation of a 3000-to 4000-liter portion was started about 36 hours after collection. The centrifugate was sent to the Department of Health and Welfare for bioassay. Centrifugation of a second 3000-4000 liter batch yielded a centrifugate which was examined at ARL. The amount of suspended solids thus isolated corresponded to about 5 mg/liter in the original water sample. More than 50% of this was non-living detritus. The living material consisted mostly of pennate diatoms of the genus Nitzschia (first tentatively identified as the species Nitzschia seriata, but later shown to be Nitzschia pungens), though other plankton species were also present. Domoic acid was detected and confirmed in this solid residue, but quantitation was unreliable due to unfortunate uncertainties in sample provenance at the height of the most frenzied activity at ARL (see #C 3 and 4 below, however). A 100-liter batch of this same sample was filtered at gentle pressure (< 5 lb./in$^2$) through a 0.3-micron filter, and the retentate sent to colleagues at Dalhousie University and at the Department of Fisheries and Oceans for culturing.

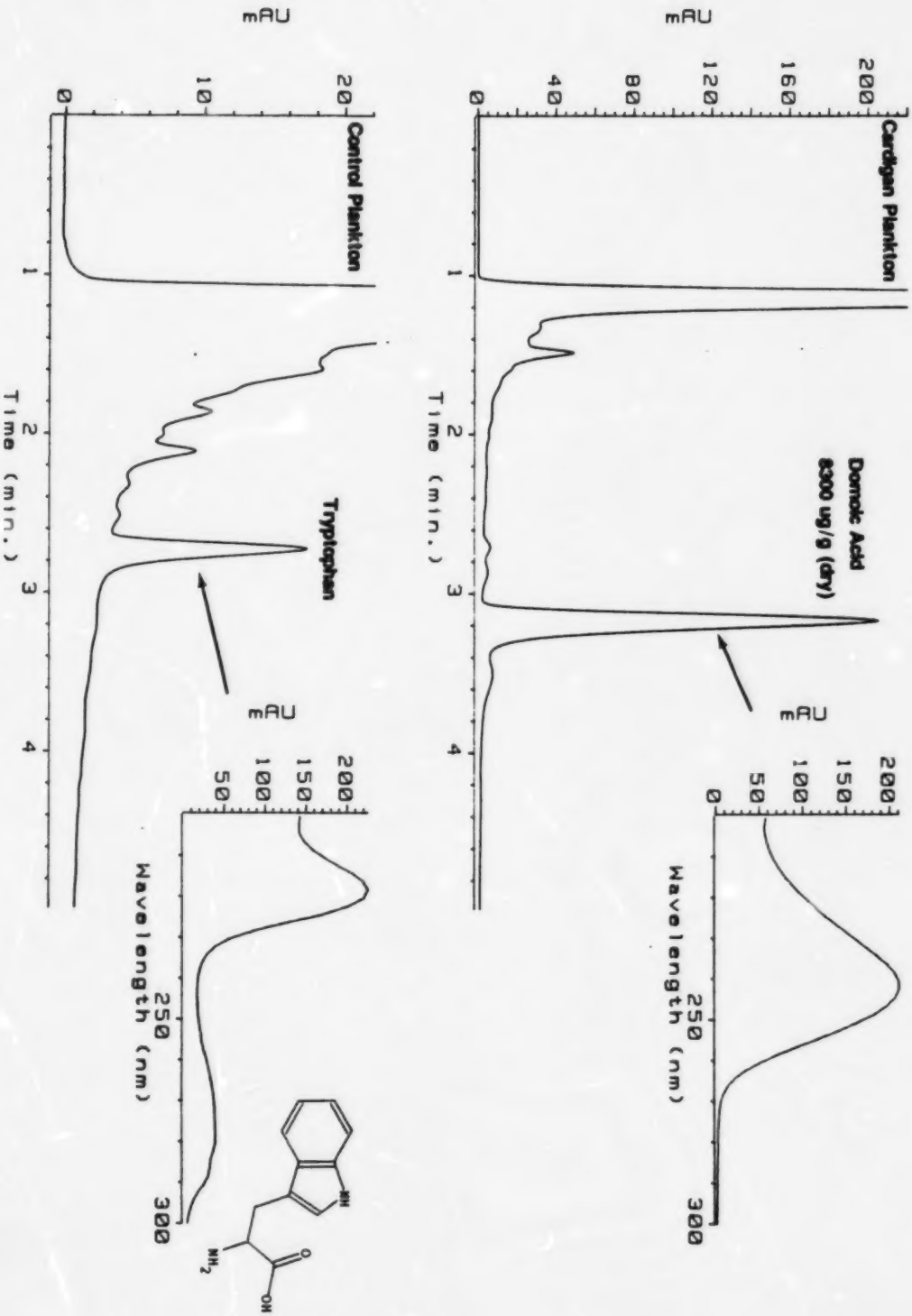
3. Plankton-tow samples were collected by L.Hanic (University of PEI) from Cardigan Bay itself and separately from about 6 miles offshore. Roughly equal weights of solids were collected in the two cases, but the off-shore sample

required about 10 times longer tow-time. This difference presumably reflects the difference between the densities of particulate matter in the two locations. Both samples were found to contain about 1% domoic acid by weight based upon dry particulate matter (Figure 16). It is important to note that the domoic acid was determined as the sum of the amounts found in the pellet and in the supernatant from the centrifuge; for one of the samples a large fraction of the total domoic acid was found in the supernatant due to extensive lysing before centrifugation. These results imply the presence of very large quantities of domoic acid in the region, whose formation could not be accounted for by any rare species (see #C1 above).

4. Water samples were collected by helicopter (arrangements by M.Bewers, DFO) from several key locations in PEI. Each sample (20-25 liters) was filtered through a 0.3 micron filter, within about 40 hours after collection. Again the samples were maintained at 0-5 deg.C without freezing. Examination by both light and electron microscopy confirmed

Figure 16. Comparison of analyses, by the fast ARL procedure, of plankton samples obtained by tow techniques in Cardigan Bay and in a control region.

FIGURE 16


Cardigan Plankton
Domoic Acid
8300 ug/g (dry)
Control Plankton
Tryptophan
mAU
Time (min.)
Wavelength (nm)
NH
NH2
OH
O

the nature of the suspended particulate matter described in #C 2 above. A control sample was also collected from the Atlantic shore of Nova Scotia. Chemical analysis of the retentates on the filters indicated levels of domoic acid in the PEI sample ranging from 0.2-3.4 microgram/liter in the water, assuming that all the domoic acid was retained on the filter. Analysis of the filtrates will require concentration enrichments of at least 1000-fold and has not been attempted as yet. The control (Nova Scotia) sample contained no detectable domoic acid (detection limit < 2 nanogram/liter).

5. As part of the program directed to determining the source of the domoic acid, tissues of control and toxic mussels were examined by light and electron microscopy. Transmission electron micrographs taken by Dr.C.Morrison (DFO, Halifax) had already revealed unusually high numbers of lysosomes in the digestive glands of toxic mussels. At ARL digestive glands were frozen, fractured and freeze-dried prior to examination in the scanning electron microscope. Large numbers of lysosomes were observed in the digestive glands of the toxic mussels as compared to the controls. At least in part this observation could be attributed to the apparent healthy nutritional state of the toxic mussels, i.e. the toxic mussels had clearly been feeding well. However it also suggested the possibility that the toxin might be sequestered in the lysosomes. To this end digestive glands from both toxic and control mussels were fractionated by the technique described by Dr.M.Moore (Institute for Marine Environmental Research, Plymouth, U.K., personal

communication) for the preparation of mussel lysosomes. The results of this step-gradient ultra-centrifugation of the tissue extract indicated that 30% of the domoic acid was bound in the cell stroma, debris and nuclei, centrifuged down in the first spin. Subsequently some 60% was found in the particulate-free soluble fraction. Only some 5% was found in the primary lysosome fraction and no domoic acid was detected in the secondary and tertiary lysosome fractions. These results indicated that the majority of the domoic acid existed free in the mussel cytoplasm, although it is not possible to completely rule out the possibility that some of this free domoic acid may have arisen from lysosomes ruptured during the fractionation process. These findings are of intrinsic interest, but did not provide information helpful to the problem of the source of the domoic acid.

6. At an early stage of the investigation, mussels were examined for the population of fungi associated with them. Several fungi were identified, and all of these were typical of those normally found in muds and soils. More importantly no significant differences were apparent between the fungal species associated with toxic and control mussels, although the sample was too small for any definite conclusion to be reached. However this line of investigation was not pursued further.

## CONCLUDING REMARKS

This report constitutes a "historical snapshot" of progress made on the P.E.I. mussel toxin problem up to mid-January, 1988. Considerable additional work on the characterisation of domoic acid has been completed in the five months since then. Despite all of this additional work the conclusions reported to the Mussel Toxin Working Group on 12 Jan., 1988, as summarised above, have remained unaltered in all important respects. In particular the conclusion that all of the mussel toxicity can be attributed to domoic acid has survived unscathed.

The Department of Fisheries and Oceans declared the affected regions cleared for harvesting of molluscs in stages, e.g. see DFO News releases NR-G-002 (12 Jan., 1988), NR-G-88-05 (28 Jan., 1988) and NR-G-88-10 (5 April, 1988); the last of these cleared the Cardigan River, the worst affected area in eastern P.E.I.

Manuscripts describing work on chemical and spectroscopic properties of domoic acid, and which have been submitted for publication in the open scientific literature, include the following:

1. "Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern PEI".
J.L.C.Wright, R.K.Boyd, A.S.W.de Freitas, M.Falk, R.A.Foxall, W.D.Jamieson, M.V.Laycock, A.W.McCulloch, A.G.McInnes, P.Odense, V.Pathak, M.A.Quilliam, M.Ragan,

P.G.Sim, P.Thibault, J.A.Walter, M.Gilgan, D.Richard and D.Dewar.

Submitted to Canad.J.Chem.

2. "High Performance Liquid Chromatography of domoic acid, a marine neurotoxin."
M.A.Quilliam, P.G.Sim, A.W.McCulloch and A.G.McInnes.

Submitted to Analyt.Chem.

3. "Liquid Chromatographic determination of domoic acid in shellfish products using the AOAC paralytic shellfish poison extraction procedure."
J.F.Lawrence, C.F.Charbonneau and C.Menard (Natl.Health and Welfare, Ottawa), M.A.Quilliam and P.G.Sim.

Submitted to J.Chromatog.

4. "The infra-red spectrum of domoic acid."
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Submitted to Biomed.Env.Mass Spectrom.

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Other investigations of chemical characterisation of domoic acid and related compounds are in progress, but are not yet ready for submission to refereed journals.

In addition a major effort devoted to identifying sources of domoic acid in the waters around P.E.I. is still in progress.

## APPENDIX 1.

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## APPENDIX 2

### PHARMACOLOGY OF DOMOATE AND KAINATE AND IMPLICATIONS FOR SHELLFISH TOXICITY

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I. Introduction. This report deals with the pharmacology and toxicology of domoic acid and the related excitatory amino acid, kainic acid. Unfortunately, the literature on domoic acid is sparse. However, as will be seen in the following discussion, domoic acid probably acts in a manner identical to kainic acid and the latter has been studied extensively. Both domoate and kainate are found in certain marine algae and it is likely that problems associated with contamination of shellfish with domoate can apply equally to kainate. Much of the information presented in this report has been synthesized from two recent reviews (Advances in Experimental Medicine and Biology, 203, 1986; Trends in Neuroscience 10, No. 7, 1987).

II. Description of the toxic syndrome in humans who have ingested contaminated shellfish. This description has been provided by the NRC laboratory in Halifax from information obtained from NHW in Ottawa. Patients have presented with: 1) short-term memory. I have not been informed of the sequence with which these symptoms appeared. The type of epilepsy has not been made public but if it resembles limbic (temperal lobe, psychomotor) epilepsy then all of the above symptoms

would be consistent with the short and longterm effects of domoic acid acting at specific kainate receptors in the central nervous system (CNS), particularly the hippocampus.

III. Description of the toxic syndrome in mice following injection with extracts of contaminated mussels. Dr. Wright of the NRC informs me that mice injected i.p. with extracts of contaminated mussels exhibit bilateral synchronized forelimb scratching movements, trembling, hindlimb extensions and ultimately death, the latter probably due to asphyxiation. I have described these symptoms to Dr. H. Robertson who studies limbic seizures and he says that these behaviours are characteristic of limbic seizures in rodents. The toxic compound in contaminated mussels has been isolated and identified by the NRC laboratory as domoic acid. Moreover, Dr. Wright tells me i.p injections of domoate, kainate and mussel extracts produce essentially similar effects in mice, domoate being about 10 times more potent than kainate. These results argue strongly for the toxic substance in contaminated shellfish being domoic acid.

IV. Receptors for excitatory amino acids in the CNS. There is little evidence that excitatory amino acids play a significant role in neurotransmission in the peripheral nervous system with the exception of the retina. However, glutamate and aspartate are important neurotransmitters in the CNS. These endogenous excitatory neurotransmitters act at 3 separate, pharmacologically identifiable receptors in the CNS: 1) the NMDA receptor, 2) the kainate receptor, and 3) the quisqualate receptor. Both domoate and kainate are specific agonists (i.e. produce activation) at kainate receptors. The relative

potencies of domoic versus kainic acid as agonists at these receptors vary depending on the system studied but domoic acid usually appears to be at least as potent as kainic acid and may be 2-3 times more potent in electrophysiological studies in vitro (Coyle, 1983). It should be pointed out that their relative potencies in vivo following ingestion could be affected by numerous factors including differential absorption from the gastrointestinal tract, hepatic metabolism, excretion by the kidney, and capacity to cross the blood-brain barrier. Unfortunately, I am not aware of any studies concerning the absorption, distribution, metabolism and excretion of domoic acid.

Activation of kainate receptors results in increases in $Na^+$ and possibly $K^+$ conductances and the consequent depolarization of neurones (Cherubini et al., 1986; Smart et al., 1886; MacDermott and Dale, 1987). Kainate receptors are localized in discrete regions of the CNS including the stratum lucidum of CA3 in the hippocampus (the region innervated by the mossy fibres which originate in the granular cell layer), the dentate gyrus of the hippocampus, the amygdala, layers V and VI of the cerebral cortex, the hypothalamus, the reticular nucleus of the thalamus, the caudate putamen (nigrostriatal pathway), the granule cell layer of cerebellum, and ventrally-located, motor-associated nuclei in the mammillary bodies and the pons (Cotman and Monaghan, 1986; Cotman et al, 1987).

The high density of kainate receptors in the hippocampus and amygdala is particularly relevant to this discussion because these regions have been implicated in limbic seizures and memory and, as is

discussed in detail below, systemic administration of kainic acid in rats produces limbic status epilepticus-type seizures and patterns of degeneration in the hippocampus which resemble those observed in postmortem biopsies of humans suffering from temporal lobe epilepsy (Babb, 1986). Moreover, kainic acid-induced lesions of the nigrostriatal pathway have been used to produce an animal model of Huntington's chorea, which is associated with abnormal movements, thought disorders and memory deficits (Coyle, 1983).

Unfortunately, unlike NMDA receptors, there are no known specific antagonists of kainate receptors currently available. Specific kainate receptor antagonists could provide a means of treating some of toxic effects of domoate and kainate.

V. Effects of systemically administered kainic acid. When 8-12 mg/kg kainic acid is injected systemically i.v., i.p. or s.c. into rats, limbic seizures are produced. Doses less than 1 µg injected directly into the CNS produce similar effects. This suggests a central site of action and poor permeability of the blood-brain barrier to kainate. Berger et al. (1986) have estimated that the free kainate levels in the brain following systemic administration would be less than 1µM but sufficient to saturate specific kainate receptors. One hour following injection (a few minutes after onset of generalized seizures) scattered neurones in the hippocampus appear dark and condensed, with some swelling of mitochondria and endoplasmic reticulum and the neuropil appears microvasculated indicating edema (Lassmann et al., 1986). During the following hours, neuronal and dendritic changes

proceed and astrocyte swelling increases dramatically. Extreme vacuolation is observed in those regions of hippocampus known to possess high densities of kainate receptors and some damage to the blood-brain barrier becomes evident. After 1 day, necrosis of neurones, myelin and oligodendrocytes is apparent. Damage is also observed in the amygdala, pyriform cortex and thalamic nuclei (Nitecka and Tremblay, 1986).

There has been some controversy concerning the exact cause of these longterm, degenerative effects of kainate. Coyle (1983) has argued that the degenerative effects of kainate are not related specifically to seizuring, based largely on the observation that NMDA produces more profound seizure activity than does kainate but is less neurotoxic. However, most authors support the concept that the neuronal degeneration is due to excessive neuronal stimulation, the so-called "excitotoxic effect". Indeed, prevention of kainate-induced seizure activity with diazepam, baclofen or phenobarbital attenuates neuronal degeneration in the rat brain (Olney, 1986; Nadler et al., 1986). The toxicity appears to be indirect and related to excessive stimulation of excitatory pathways in the regions affected.

It has been suggested that kainate may release the excitatory neurotransmitter glutamate and possibly potentiate its action postsynaptically. Some authors have suggested that increased influx of $Ca^{2+}$ into neurones may produce degeneration (Coyle, 1983), while others suggest that toxicity is dependent on $Na^{+}$ and possibly $Cl^{-}$ influx rather than $Ca^{2+}$ influx (Olney, 1986). It has been suggested that the

release of zinc from nerve terminals may also play a role (Chung et al., 1986). Finally, it has been proposed that disruption of the blood-brain barrier and consequent entrance of proteins into the brain may play a part (Ruth, 1986), although the disruptions to the blood-brain barrier are less than those observed with encephalitis or osmotic opening of the blood-brain barrier (Lassman et al., 1986).

VI. Possible explanations as to why fewer than 30 of the thousands who ate contaminated mussels were seriously affected. The most obvious possibility to explain this would be that some of the contaminated mussels contained much higher levels of domoic acid than did others so that only the individuals who ingested those specific mussels would have obtained toxic doses.

I have not found any literature concerning the absorption, metabolism, distribution and elimination of domoic acid but these aspects could provide possible explanations as to why some individuals were more affected than others. Domoic acid would first have to be absorbed from the gastrointestinal tract. There are specific transport systems for many amino acids in the intestine. If domoic acid is taken up by one of these systems, it is possible that the capacity of such a system could vary from individual to individual or be affected by competing dietary acidic amino acids. It is also possible that domoic acid is absorbed by passive diffusion across the cell membrane of mucosal cells and the rate of absorption would then depend on the degree of ionization of the molecule, the ionized form diffusing across the lipid barrier better than the ionized form. One would expect

domoic acid to be less ionized in an acidic environment so that ingestion of an acidic meal or drink could enhance its absorption from the gut.

Following absorption, domoic acid would be carried to the liver by the hepatic portal system where it could undergo metabolism. It is possible that certain individuals, such as those with hepatic diseases, may not metabolize domoic acid as efficiently as others. Domoic acid may be excreted by the kidney and this could be compromised in persons suffering from renal impairment. Moreover, excretion could be impaired if the urine is acidified, since this would promote the unionized form of the molecule and consequently tubular reabsorption.

By analogy to kainate, it seems likely that the blood-brain barrier prevents the entrance of most of the systemic domoic acid into the CNS. Disruption of this barrier would permit excessive amounts of domoic acid to enter the CNS.

Drugs such as caffeine and theophylline have been shown to intensify and prolong limbic seizures in kindled rats (Dragunow, 1986; Ault and Wang, 1986; Dragunow and Robertson, 1987). The methylxanthines, which are widely used (caffeine recreationally and theophylline for the treatment of asthma), are antagonists at inhibitory adenosine receptors in the hippocampus and amygdala. The combination of excitation by domoate and diminished inhibition by the methylxathines could produce more profound stimulation of the limbic regions in those individuals who have been taking methylxanthines. This could promote the development of neurotoxicity.

Finally, it is worth noting that only a small proportion of the population is susceptible to so-called "Chinese restaurant syndrome" which is due to glutamate added to certain foods as a flavour enhancer. Certain individuals may be particularly sensitive to glutamate agonists and it would be very interesting to see whether those seriously affected by shellfish poisoning had any prior history of this syndrome.

The fatalities appear to have involved primarily the elderly. This could relate to general ill health or to medical complications often associated with the elderly. In general, the elderly eliminate drugs from their bodies much slower than younger people.

VII. Possible treatments for affected individuals. 1) The prognosis for individuals who exhibit persistent symptoms following the ingestion of domoate is not good since this may indicate the development of permanent lesions in the CNS. Patients exhibiting persistent limbic epilepsy should be treated with drugs appropriate for that disease (eg. drugs for complex partial seizures such as carbamazepine or phenytoin).

2) For treatment of domoate poisonings in the early stages, there are several courses of action which may be taken. If it is determined that domoate is eliminated primarily by renal excretion, then alkalinizing the urine could hasten its elimination. As discussed above, the prevention of seizuring by treating rats with benzodiazepines, phenobarbital or baclofen diminishes the extent to which permanent kainate-induced lesions of the hippocampus develop.

Consequently, it would seem prudent to treat suspected domoate poisonings promptly with one of the above drugs in the hope that this may curtail the development of permanent lesions. Finally, new specific kainate receptor antagonists may be developed in the future and these could block the action of excitatory amino acids at kainate receptors and prevent the toxic effects of these agents.

VIII. Conclusions and recommendations. 1) The toxic syndrome observed in humans who have ingested contaminated mussels appears consistent with that observed in rats treated with kainate receptor agonists and is probably due to domoic acid.

2) Initial therapy might involve prompt treatment with benzodiazepines, phenobarbital or baclofen to prevent seizures and the consequent development of permanent central lesions. Persistent symptoms of limbic epilepsy should be treated with carbamazepine or phenytoin.

3) Because there is a vast literature dealing with the toxic effects of kainate in the rat, this species should be used in all subsequent screening programs for domoate and kainate contamination of shellfish.

4) Research should be conducted to determine the short and longterm effects of domoate in the CNS of rats. The absorption, distribution, metabolism and excretion of domoate should also be ascertained; results from these studies may yield information which could provide means of diminishing domoate absorption or hastening its elimination.

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